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RESEARCH PAPER

Histamine modulates γδ-T lymphocyte migration and cytotoxicity, via G_i and G_s protein-coupled signalling pathways

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Background and purpose: The biogenic amine, histamine plays a pathophysiological regulatory role in cellular processes of a variety of immune cells. This work analyses the actions of histamine on γδ-T lymphocytes, isolated from human peripheral blood, which are critically involved in immunological surveillance of tumours.
Experimental approach: We have analysed effects of histamine on the intracellular calcium, actin reorganization, migratory response and the interaction of human γδ T cells with tumour cells such as the A2058 human melanoma cell line, the human Burkitt's Non-Hodgkin lymphoma cell line Raji, the T-lymphoblastic lymphoma cell line Jurkat and the natural killer cell-sensitive erythroleukaemia cell line, K562.
Key results: γδ T lymphocytes express mRNA for different histamine receptor subtypes. In human peripheral blood γδ T cells, histamine stimulated *Pertussis* toxin-sensitive intracellular calcium increase, actin polymerization and chemotaxis. However, histamine inhibited the spontaneous cytolytic activity of γδ T cells towards several tumour cell lines in a cholera toxin-sensitive manner. A histamine H_4 receptor antagonist abolished the histamine induced γδ T cell migratory response. A histamine H_2 receptor agonist inhibited γδ T cell-mediated cytotoxicity.
Conclusions and implications: Histamine activated signalling pathways typical of chemotaxis (G_i protein-dependent actin reorganization, increase of intracellular calcium) and induced migratory responses in γδ T lymphocytes, via the H_4 receptor, whereas it down-regulated γδ T cell mediated cytotoxicity through H_2 receptors and G_s protein-coupled signalling. Our data suggest that histamine activated γδ T cells could modulate immunological surveillance of tumour tissue.
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Keywords: histamine; γδ T lymphocytes; migration; cytotoxicity; G protein

Abbreviations: CI, chemotactic index; dimaprit, S-(3-dimethylaminopropyl)isopthiourea; FITC, fluoro-isothiocyanate; H_1, H_2, H_3, H_4, histamine receptor subtypes; HTMT, 6-[2-(-imidazolyl)ethylamino]-N-(4-trifluoromethylphenyl) heptanecarboxamide dimaltate; IL, interleukin; NK, natural killer; thioperamide, N-cyclohexyl-4-(1H-imidazol-4-yl)-1-piperidinecarbothioamide maleate salt (thioperamide)

Introduction

γδ T cells are a population of lymphocytes expressing functional γδ T cell receptor (TCR) genes (Brenner *et al.*, 1986). This subtype of T cells, presumably an ancient type of lymphocytes, is derived from haematopoietic stem cells that share certain characteristics with other immune cells, such as antigen presentation, immune modulatory properties and cytolytic activity (Nakata *et al.*, 1990; Girardi, 2006). Two main subsets of γδ T cells are distinguished according to their location. Resident γδ T cells are found in skin, uterine and epithelial tissues, whereas circulating/systemic γδ T cells can be isolated from peripheral blood or lymphoid tissues (Kabelitz, 1993; Chen, 2002).

In contrast to αβ T lymphocytes, γδ T cells do not need antigens presented on classical MHC-molecules for

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recognition (Kabelitz *et al.*, 2000). Instead, they recognize antigens bound to CD1 molecules. They are able to recognize a number of natural phosphoantigens derived from plants, bacteria, protozoa and viruses, as well as endogenous ligands derived from tumours (Bukowski *et al.*, 1995; Bauer *et al.*, 1999; Boullier *et al.*, 1999; Selin *et al.*, 2001). Several lines of evidence involve γδ T cells in primary host defence as well as in tumour surveillance; they are also known to attack bacterial and virus-bearing cells as well as transformed cells (Wrobel *et al.*, 2007). This cytotoxic activity is mediated by production and release of perforin and granzymes (Nakata *et al.*, 1990; Girardi, 2006). The antitumour activity of γδ T cells either is mediated via endogenous ligands in γδ TCR-dependent fashion or depends on the interaction of the cells with the natural killer (NK) cell receptor, NKG2D (Bukowski *et al.*, 1995; Wrobel *et al.*, 2007).

Histamine (β-imidazolylethylamine) is a biogenic amine, stored in the granules of tissue mast cells, blood basophils and neural cells (Riley and West, 1952; Kinet, 1999). It is involved in different physiological and pathological responses, such as the immune response, gastric acid secretion, neurotransmission and angiogenesis (Brimble and Wallis, 1973; Akdis and Simons, 2006; Hegyesi *et al.*, 2008; Yakabi *et al.*, 2008; Zampeli and Tiligada, 2009). The expression of the enzyme that forms histamine, histidine decarboxylase, in several leukaemia and highly malignant forms of cancer, such as melanoma, small cell lung carcinoma and breast adenocarcinoma tumour, suggests that histamine plays a functional role in the pathogenesis of various types of cancer (Matsuki *et al.*, 2003; Sonobe *et al.*, 2004; Aichberger *et al.*, 2006; Hegyesi *et al.*, 2008).

Histamine is known to regulate humoral and cellular immunity by controlling the production of pro-inflammatory cytokines, the expression of adhesion molecules and the migration of inflammatory cells such as eosinophils, dendritic cells, NK cells and αβ T cells (Gutzmer *et al.*, 2005; Damaj *et al.*, 2007). The pleiotropic effects of histamine are mediated by four types of receptors (histamine H_1, H_2, H_3 and H_4; nomenclature follows Alexander *et al.*, 2008). They belong to the serpentine (7-TM) receptor superfamily and couple to different types of G proteins; these proteins initiate distinct intracellular signalling pathways (Hill *et al.*, 1997). Histamine H_1 receptors preferentially couple to $G_{q/11}$ proteins to mediate the mobilization of intracellular Ca^{2+} as well as the activation of protein kinase C, extracellular signal regulated kinase and the transcription factor, nuclear factor κB (Matsubara *et al.*, 2005). The H_2 receptor interacts with G_s proteins and stimulates cAMP accumulation (Baker, 2008). H_3 receptors are primarily expressed in the brain, inhibit cAMP formation and regulate intracellular Ca^{2+} transients (Drutel *et al.*, 2001). The H_4 receptor regulates intracellular Ca^{2+} mobilization and chemotaxis in mast cells, eosinophils and NK cells via *Pertussis* toxin-sensitive G_i proteins (Damaj *et al.*, 2007; Leurs *et al.*, 2009).

In the current work we examine the biological activity of histamine in γδ T cells. We provide evidence for functional expression of histamine H_2 and H_4 receptors and show that histamine induces intracellular Ca^{2+} transients, actin polymerization and chemotaxis via H_4 receptors, whereas H_2 receptors promote cAMP accumulation and down-regulate the cytotoxicity of γδ T cells towards different tumour cell lines.

Methods

Preparation of γδ T cells

The use of human cells was approved by the Research Ethics Board of the University of Jena. Peripheral blood mononuclear cells were isolated using the Ficoll separation protocol (Haas *et al.*, 1993). Briefly, a density gradient centrifugation of buffy coats was performed. The leukocyte-containing pellet was resuspended in phosphate-buffered saline pH 7.2, supplemented with 0.5% bovine serum albumin and 2 mM EDTA, and the cells were labelled with an anti-TCR γδ hapten-antibody and anti-hapten micro-beads-fluoro-isothiocyanate (FITC) antibody. Labelled cells were separated with magnetic separation columns. Positive selected γδ T cells were cultured for 7–10 days in the presence of Phaseolus vulgaris phytohemagglutinin (PHA) ($2\ \mu g \cdot mL^{-1}$) for 3 days and interleukin (IL)-2 ($100\ IU \cdot mL^{-1}$) until day 7 (Nakata *et al.*, 1990; Argentati *et al.*, 2003; Wrobel *et al.*, 2007).

mRNA isolation, reverse transcription and polymerase chain reaction (RT-PCR) analysis

mRNA was isolated from 1×10^6 human peripheral blood γδ T cells using High Pure RNA isolation Kit. Fast Start Taq cDNA Polymerase Kit and Fast Start Taq DNA Polymerase Kit were used to obtain cDNA and PCR products. The primers were designed to recognize sequences specific for each target cDNA:

H_1R (403 bp): sense 5′-CATTCTGGGGGCCTGGTTTCTCT-3′
antisense 5′-CTTGGGGGTTTGGGATGGTGACT-3′
H_2R (497 bp): sense 5′-CCCGGCTCCGCAACCTGA-3′
antisense 5′-CTGATCCCGGGCGACCTTGA-3′
H_3R (589 bp): sense 5′-CAGCTACGACCGCTTCTTGTC-3′
antisense 5′-GGACCCTTCTTTGAGTGAGC-3′
H_4R (396 bp): sense 5′-GGTACATCCTTGCCATCACATCAT-3′
antisense 5′-ACTTGGCTAATCTCCTGGCTCTAA-3′
β_2-micriglobuline (259 bp):
5′-CCTTGAGGCTATCCAGCGTA-3′
antisense 5′-GTTCACACGGCAGGCATACT-3′

Mobilization of intracellular Ca^{2+}

Intracellular free Ca^{2+} was measured in Fura-2-labelled γδ T cells using the digital fluorescence microscope unit Attofluor (Zeiss, Oberkochen, Germany) (Panther *et al.*, 2001).

Filamentous (f) actin measurements

Samples of stimulated γδ T cells (10^6 per mL; 50 μL per sample) were fixed in a 7.4% formaldehyde buffer and mixed with the staining mixture containing 7.4% formaldehyde, 0.33 μM NBD-phallacidin and $1\ mg \cdot mL^{-1}$ lysophosphatidylcholine. The fluorescence intensity was measured by flow cytometry. The relative f-actin content was compared with unstimulated controls (Lagadari *et al.*, 2009).

Migration assay

The chemotaxis of human peripheral blood γδ T cells was performed in 48-well-Microtechnic chambers from Neuro

Probe (Gaithersburg, MD, USA). Wells in the bottom of the chamber were filled with 29 μL medium containing the indicated concentration of stimulus. Over this filled chamber, a polycarbonate membrane (thickness 10 μm, diameter of the pores 8 μm) and a gasket made of silicone were fixed. The device was screwed on the top of the gasket and the cells were added in 29 μL per well (resuspended in a concentration of 1×10^5 per mL) in the upper wells of the chamber. For the assay, the chamber was incubated for 240 min at 37°C. The non-migrating cells from the wells of the upper chamber were removed after the incubation period; the filter and gasket were then removed and the cells from the bottom chamber were collected, fixed in formaldehyde (3.7%) and counted by flow cytometry. A chemotactic index (CI) was calculated as the ratio between stimulated and random migration.

In vitro *cytotoxicity assay*

Cytotoxicity was determined with a standard ^{51}Cr release assay. Target cells were labelled at 37°C for 1 h with 100 μCi $Na_2{}^{51}CrO_4$. Cells were washed and resuspended at a cell density of 1×10^6 cells·mL^{-1} in RPMI 1640 culture medium supplemented with 2% fetal calf serum. Effector and target cells at different ratios (10:1, 5:1 and 2.5:1) were placed into individual wells of 96-well plates in a total volume of 200 μL at 37°C for 4 h. After incubation, 100 μL culture supernatant was collected from each well, mixed with MicroScint-40 cocktail and analysed with a gamma counter (Topcount™, Packard Instruments). To obtain the value of total lysis, target cells were incubated with 2% Triton-X. Percentage of specific lysis was calculated using the following formula:

$$\frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

Measurement of cAMP levels

γδ T cells (1×10^6 per mL) were fixed and permeabilized before intracellular staining was performed. The amount of intracellular cAMP in the γδ T cell preparation was determined by flow cytometry (Pepe *et al.*, 1994).

Cell lines

The A2058 human melanoma cell line, the human Burkitt's non-Hodgkin lymphoma cell line Raji, the T-lymphoblastic lymphoma cell line Jurkat, and the erythroleukaemia cell line K562 originating from patients with chronic myeloid leukaemia and blast crisis were maintained at 37°C in a 5% CO_2 incubator in RPMI 1640 supplemented with 10% fetal bovine serum, 10 U·mL^{-1} penicillin, 10 U·mL^{-1} streptomycin and 1 mM L-glutamine (Herberman, 1981).

Western blot analysis

Immunoblotting was performed by running the samples on SDS–PAGE gels (20 μg protein per lane) and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 1 h at room temperature and then incubated with the first antibody (1:2000) overnight at 4°C. After washing, membranes were incubated with HRP-conjugated secondary antibody (1:10 000) for 1 h at room temperature. Proteins were detected by ECL (Amersham).

Statistical analysis

Significant differences between means ($P < 0.05$) were determined using the non-parametric two-tailed Student's *t*-test.

Materials

RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 10 U·mL^{-1} penicillin, 10 U·mL^{-1} streptomycin, 1 mM L-glutamine and Hanks BSS was purchased from Promocell (Heidelberg, Germany); recombinant human IL-2 (Proleukine) was from Chiron (Ratingen, Germany); histamine, thioperamide malate salt (T123), *Phaseolus vulgaris* PHA, cholera toxin, *Pertussis* toxin, lysophosphatidylcholine, Triton-X, ionomycin,the H_1 receptor antagonist triprolidine, H_2 receptor antagonist cimetidine and H_3 receptor antagonist/H_4 receptor agonist clobenpropit were obtained from Sigma-Aldrich (Taufkirchen, Germany); the H_2 receptor agonist dimaprit from Biomol (Hamburg, Germany); the H_3 receptor agonist imetit and the H_1 receptor agonist 6-[2-(4-imidazolyl)ethylamino]-N-4-trifluoromethylphenyl) heptanecarboxamide dimaleate (HTMT dimaleate) from Biozol (Eiching, Germany); anti-TCR γδ hapten-antibody and anti-hapten MicroBeads-FITC antibody from Miltenyi Biotech GmbH (Bergisch Gladbach, Germany); Vg9 TCR antibody from BD Biosciences Pharmingen (Heidelberg, Germany); specific antibodies to histamine H_1, H_2, H_3 or H_4 receptors from Santa Cruz Biotechnology Inc. (Heidelberg, Germany) High Pure RNA Kit, FastStart Taq DNA Polymerase Kit from Roche Diagnostics GmbH (Mannheim, Germany); SeaKem LE agarose from Cambrex (Taufkirchen, Germany); NBD-phallacidin and the histamine H_1–H_4 receptor primers from Invitrogen GmbH (Technologiepark Karlsruhe, Germany); FURA/2AM from Calbiochem (Darmstadt, Germany); Nucleopore Track-Etch membrane filtration products from Whatman International Ltd. (Kent, UK); $Na_2{}^{51}CrO_4$ from Amersham (Freiburg, Germany); Microscint-40 from PerkinElmer (Jügesheim, Germany); cAMP antibody from Abcam (Cambridge, UK); and goat anti-mouse FITC-conjugated antibody from AL-Immunotools (Friesoythe, Germany).

Results

γδ T cells expressed histamine H_1, H_2 and H_4 receptors

Using RT-PCR analysis, the expected products for the histamine H_1, H_2 and H_4 receptor subtypes were detected in γδ T cells, isolated from human peripheral blood. In contrast, the H_3 receptor was undetectable (Figure 1A). Omitting reverse transcriptase, no amplification products were observed in γδ T cells (data not shown). Expression of the H_1, H_2 and H_4 receptor subtypes were detected at the protein level by Western blot analysis (Figure 1B).

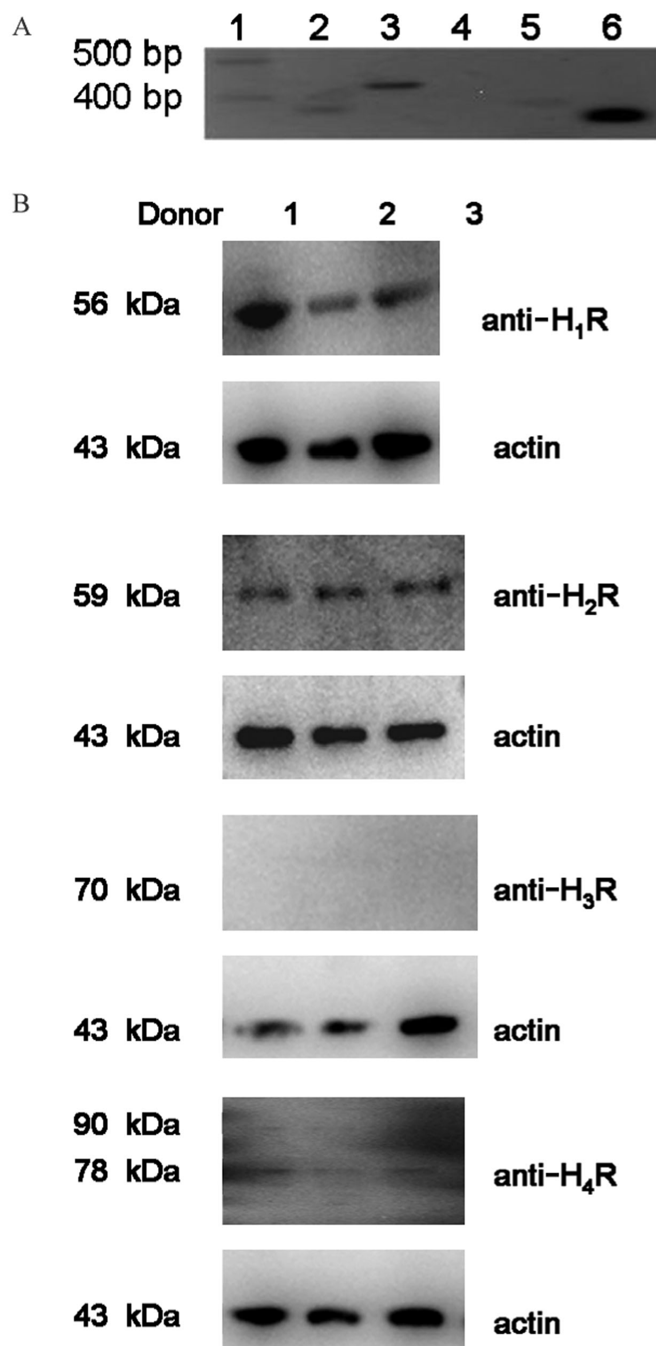

Figure 1 Expression of mRNA of histamine H_1, H_2 and H_4 receptors in human peripheral blood γδ T lymphocytes. (A) γδ T cells were isolated from human peripheral blood and expression of mRNA for histamine receptors was analysed. Lane 1 DNA molecular weight marker XIV (100–1500 bp); lane 2 H_1 (403 bp); lane 3 H_2 (497 bp); lane 4 H_3 (589 bp); lane 5 H_4 receptors (396 bp); lane 6 β_2-microglobulin (259 bp). (B) γδ T cells were isolated from human peripheral blood and expression of different histamine receptor subtypes were analysed by Western blot analysis. Lane 1 H_1 receptors (56 kDa); lane 2 H_2 receptors (59 kDa); lane 3 H_3 receptors (70 kDa); lane 4 H_4 receptors (78/90 kDa), actin (43 kDa). Experiments were repeated three times with identical results.

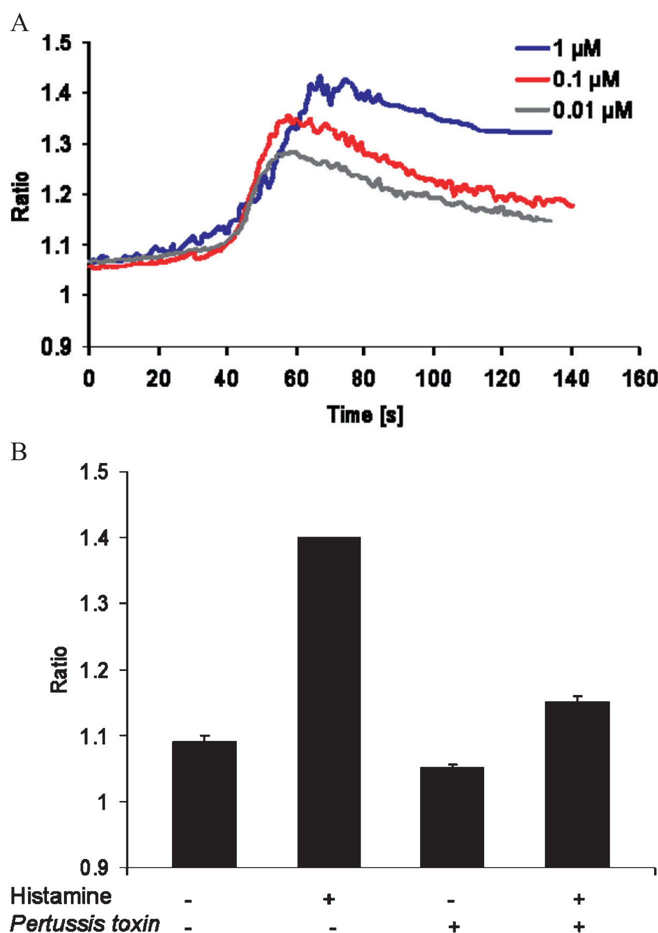

Figure 2 Histamine induces calcium transients in human γδ T lymphocytes. (A) Cells were loaded with Fura-2/AM and stimulated with 0.01 µM, 0.1 µM and 1 µM histamine. Intracellular Ca^{2+} transients were followed by digital fluorescence microscopy and ratio between 340 nm and 380 nm was calculated. Representative data from one experiment are shown. Experiments were repeated three times with identical results. (B) Cells were pre-incubated with or without *Pertussis* toxin (100 $ng \cdot mL^{-1}$) for 90 min at 37°C and stimulated with 1 µM histamine. Calculated ratio after stimulation with and without histamine is shown. Data are means of three different experiments of three different donors ± SEM.

Histamine induces actin polymerization, intracellular Ca^{2+} mobilization and chemotaxis in γδ T cells through Pertussis toxin-sensitive G_i proteins

Histamine induces Ca^{2+} transients in different types of leukocytes (Feske, 2007) and in our experiments, histamine induced a rapid and concentration-dependent intracellular response in human γδ T cells (Figure 2A). Ca^{2+} transients are mainly caused by mobilization of Ca^{2+} from intracellular stores or by their influx across the plasma membrane from the medium. In order to determine which of these two mechanisms was involved, experiments in the presence of EGTA in the medium were performed. Pre-incubation of γδ T cells with EGTA (4 mM) did not influence the histamine-initiated Ca^{2+} intracellular rise, implying the mobilization of Ca^{2+} from intracellular stores (data not shown). To investigate the involvement of G_i proteins in this response, we took advantage of *Pertussis* toxin. This toxin uncouples G_i

proteins from serpentine receptors by ADP-ribosylation. Pretreating γδ T cells for 1 h with *Pertussis* toxin (100 ng·mL^{-1}) strongly inhibited the histamine-induced Ca^{2+} increase in these cells which in turn implied the involvement of G_i proteins (Figure 2B). To check the responsiveness of *Pertussis* toxin-treated cells, experiments with ionomycin were performed. Ca^{2+} transients induced by ionomycin were not influenced by pretreatment of cells with *Pertussis* toxin (data not shown).

Next, actin reorganization was analysed by flow cytometry. A rapid increase in f-actin content (by about 60%) was induced when γδ T cells were stimulated with histamine (Figure 3A). The response was transient and reversible with maximal values within 30 s. To test the participation of G_i proteins in this response, γδ T cells were also pre-incubated with *Pertussis* toxin before being exposed to histamine (Figure 3B). Pretreating γδ T cells with *Pertussis* toxin almost completely abolished the effect of histamine on actin polymerization. In contrast, cells pretreated with cholera toxin did not differ significantly from control cells (Figure 3C).

Intracellular Ca^{2+} transients and actin reorganization are prerequisites for cell migration. Therefore, human peripheral blood γδ T cells were exposed to different concentrations of histamine (0.01 μM–1 μM), and migration in the Boyden chambers was evaluated. Histamine induced the typical bell-shaped concentration dependent chemotactic response of γδ T lymphocytes (Figure 4A). Maximal chemotactic responses were observed upon stimulation with 0.01 μM histamine. Moreover, histamine-stimulated migration was also abolished by pretreating γδ T cells with *Pertussis* toxin (100 ng·mL^{-1}) (Figure 4B).

To determine the subtype of histamine receptor involved in the histamine-induced chemotactic response in human γδ T cells, we used receptor-selective agonists and antagonists. The histamine H_1 receptor agonist HTMT, the H_2 receptor agonist dimaprit and the H_3 receptor agonist imetit did not induce any significant change in chemotactic activity (Figure 5A), whereas γδ T cells exposed to the H_4 receptor agonist clobenpropit showed migration comparable to that after histamine in these cells. Pretreating γδ T cells with the H_4 receptor antagonist thioperamide prevented histamine-induced migration (Figure 5B), suggesting that in γδ T cells, migration in response to histamine occurs specifically through the H_4 receptors.

Histamine-induced intracellular cAMP levels in γδ T cells

Histamine is known to affect the intracellular cAMP levels in human dendritic cells and lymphocytes via H_2 receptors and G_s proteins (Idzko *et al.*, 2002). In order to characterize the functional expression of H_2 receptors in human γδ T cells isolated from peripheral blood, intracellular cAMP levels after stimulation with histamine were determined by flow cytometry. A significant increase ($P < 0.0001$) in cAMP levels, as reflected by increases in mean fluorescence intensity (MFI) was observed 1 min after histamine treatment (Figure 6A). Moreover, cAMP levels reached a maximum after 4 min and remained high for at least 30 min after histamine stimulation (Figure 6B).

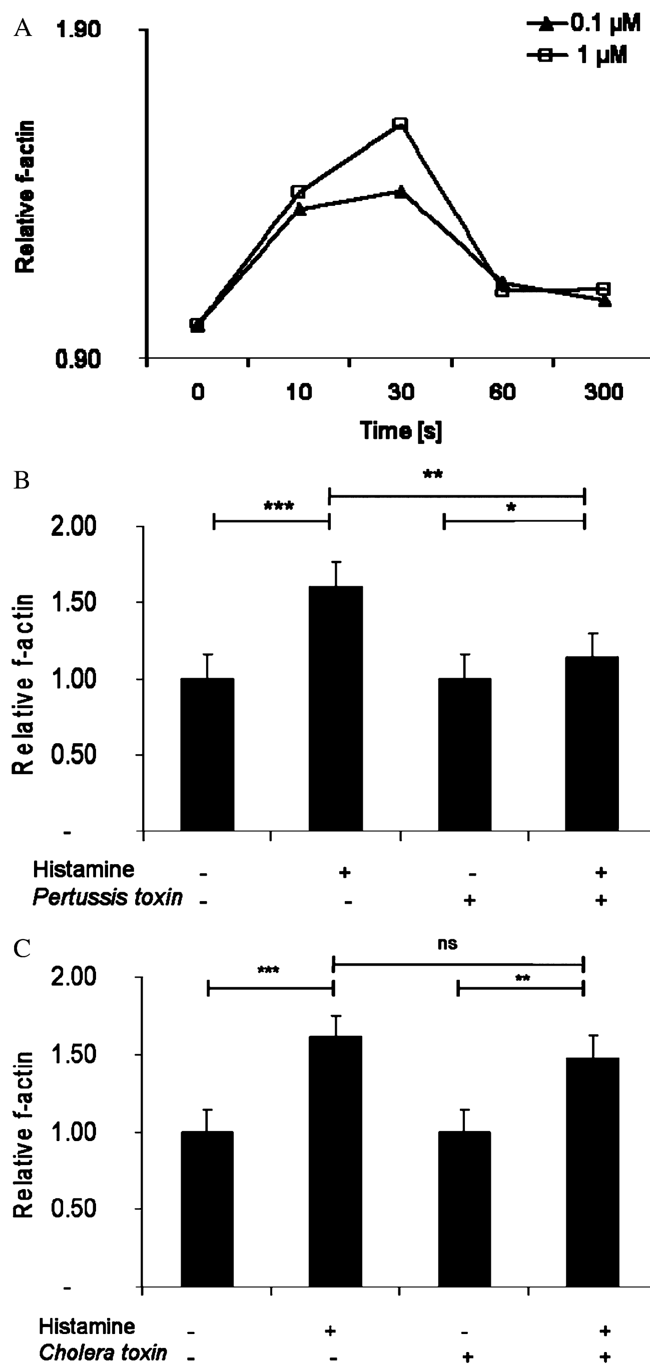

Figure 3 Effects of histamine on actin polymerization in human γδ T cells. (A) Cells were exposed to 0.1 μM–1 μM histamine and f-actin content was measured by flow cytometry. (B) Cells were pre-incubated with or without *Pertussis* toxin (100 ng·mL^{-1}) for 90 min at 37°C and stimulated with 1 μM histamine for 30 s and the increase in f-actin content was analysed. (C) Cells were pre-incubated with or without cholera toxin (0.5 μg·mL^{-1}) for 90 min at 37°C and stimulated with 1 μM histamine for 30 s and the f-actin content was analysed. (Line 1: unstimulated γδ T cells; Line 2: histamine stimulated γδ T cell; Line 3: cholera toxin pretreated γδ T cells; cholera toxin pretreated γδ T cells exposed to histamine) All data are means of three different experiments using three different donors ± SEM (***$P < 0.0001$; **$P > 0.005$; *$P > 0.05$). ns, not significant.

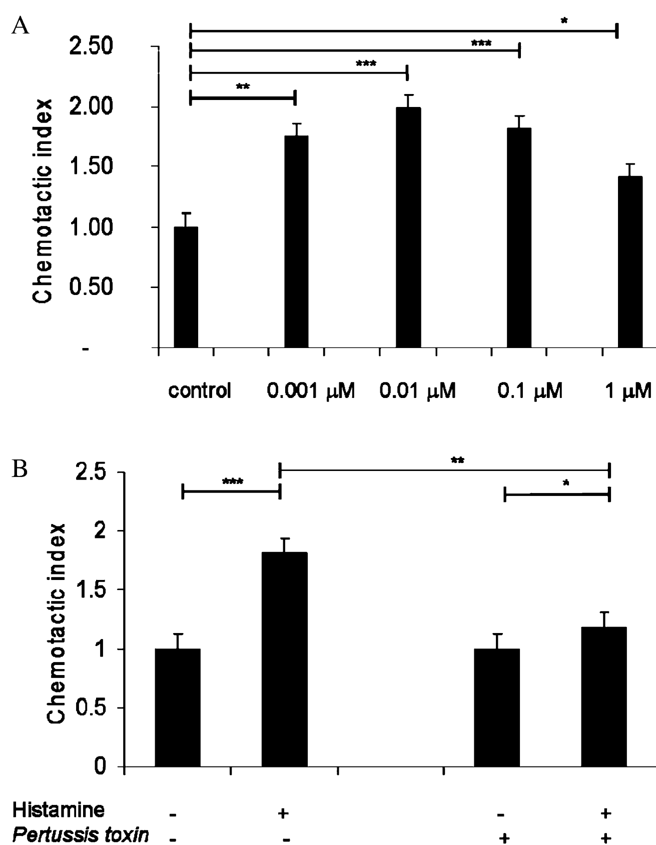

Figure 4 Effects of histamine on chemotaxis in human γδ T cells. Cells were exposed to different histamine concentrations (0.001 μM–1 μM) in Boyden chambers. Migrated cells in the bottom wells of the Boyden chamber were fixed with formalin and counted by flow cytometry. (B) γδ T cells were pretreated with *Pertussis* toxin for 1 h at 37°C and migration in response to 0.1 μM histamine was analysed. All data are means ± SEM (***$P < 0.0001$; **$P > 0.005$; *$P > 0.05$).

Histamine affects the cytotoxic activity of γδ T cells against tumour cells

We have previously shown that the activation of G_s protein coupled receptors and the up-regulation of cAMP lead to the down-regulation of cytotoxic responses in NK cells. In addition, γδ T cells are known to exhibit cytolytic activity towards different human tumour cell lines, such as the myeloid leukaemia cell line (K562), cutaneous malignant melanoma cells and the non-Hodgkin T cell line Jurkat (Sicard *et al.*, 2001; Argentati *et al.*, 2003). To better characterize the cytolytic activity of γδ T cells, *in vitro* radioactive assays were performed using different cell lines. Target cells were labelled for 1 h with chromium (100 μCi per 10^6 cells) and co-cultured for 4 h at 37°C with γδ T cells to allow spontaneous cytotoxicity. As shown in Figure 7A, although γδ T cells displayed cytolytic activity against all cell lines tested, their lytic capacity was highest against K562 cells. Therefore, this cell line was chosen for further experiments analysing the influence of histamine on the cytolytic activity of γδ T cells. Histamine significantly reduced the cytolytic capacity of γδ T cells against K562 cells, at all cell ratios (E:T) tested (Figure 7B).

In order to find out which subtype of histamine receptor modulates cytotoxicity in γδ T cells, experiments with receptor-specific agonists and antagonists were performed.

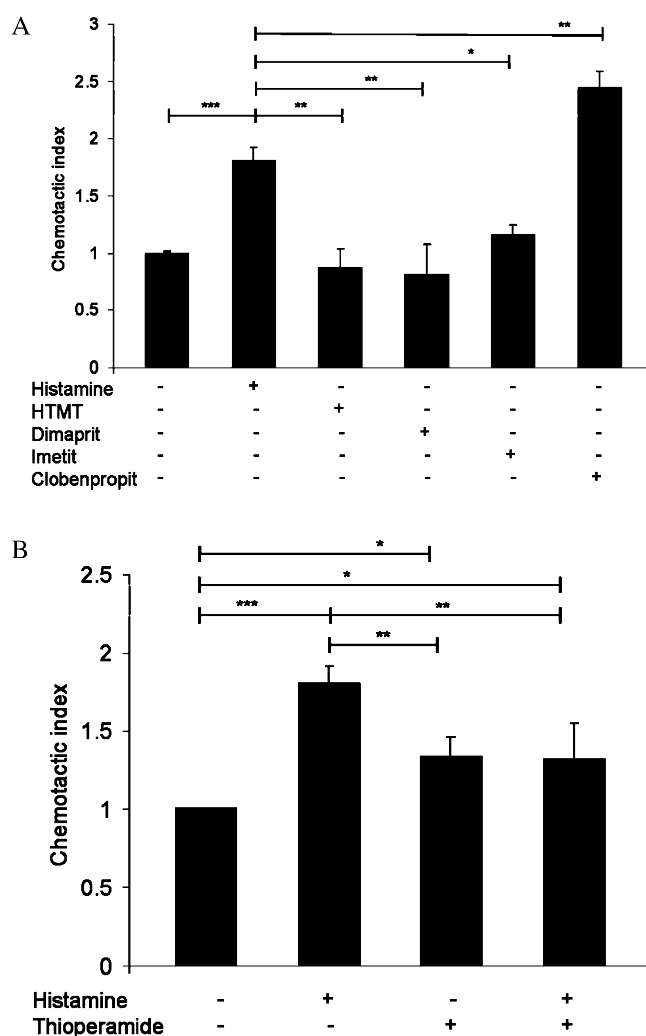

Figure 5 Effects of histamine receptor agonists and antagonist on chemotaxis in γδ T cells. (A) γδ T cells isolated from healthy donors were exposed to histamine and selective agonists – HTMT for H_1 receptors), dimaprit for H_2 receptors, imetit for H_3 receptors and clobenpropit for H_4 receptors and migration was measured. (B) γδ T cells were pretreated with the H_4 receptor antagonist thioperamide and the migration assay was performed. All data are means ± SEM ($n = 3$) (***$P < 0.0001$; **$P > 0.005$; *$P > 0.05$).

Agonists specific for H_1 or H_3 receptors did not affect the spontaneous lysis capacity of K562 cells by γδ T cells, whereas the H_2 receptor-agonist dimaprit reduced the spontaneous lysis capacity of γδ T cells against K562 cells (Figure 8A). Moreover, while the H_4 receptor antagonist did not prevent the histamine-induced effect on cytotoxicity, the H_2 receptor antagonist cimetidine abolished this effect of histamine in γδ T cells (Figure 8B). These experiments suggest that the modulatory effect of histamine on γδ T cell mediated cytotoxicity requires activation of the H_2 receptor subtype.

We next determined the involvement of different G proteins in the histamine modulation of cytotoxicity. Thus, γδ T cells were pretreated with the G_i protein inhibitor, *Pertussis* toxin and the G_s-activator, cholera toxin, and their cytotoxic activity against K562 cells examined (Figure 9). *Pertussis* toxin did not significantly alter the effect of histamine on cytotoxicity. On the contrary, pretreating γδ T cells with cholera toxin

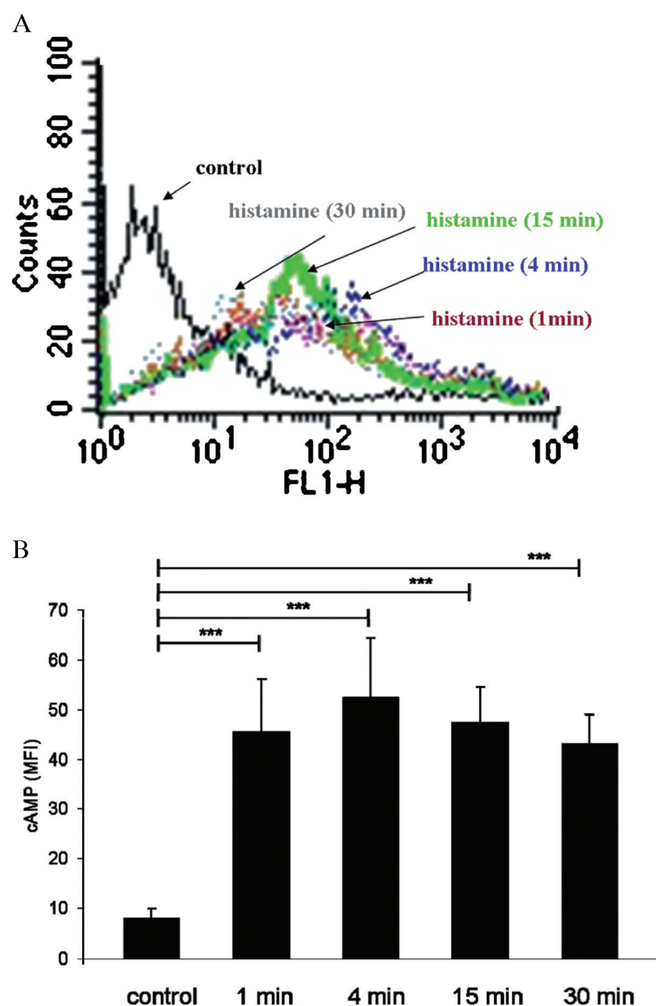

Figure 6 Effect of histamine on intracellular cAMP levels in human peripheral blood γδ T cells. (A) Distribution of fluorescence intensity in control cells and γδ T cells stimulated with 1 μM histamine for 15 min is shown. Aliquots of cells were fixed and stained as described in Methods. The fluorescence intensity was measured by flow cytometry. Representative data of one experiment are shown; experiments were performed three times in triplicate. (B) Time course of cAMP levels after stimulation with 1 μM histamine. Experiments were repeated five times with γδ T cells isolated from different donors. Data are means ± SEM (*n* = 5) (****P* < 0.0001). MFI, mean fluorescence intensity.

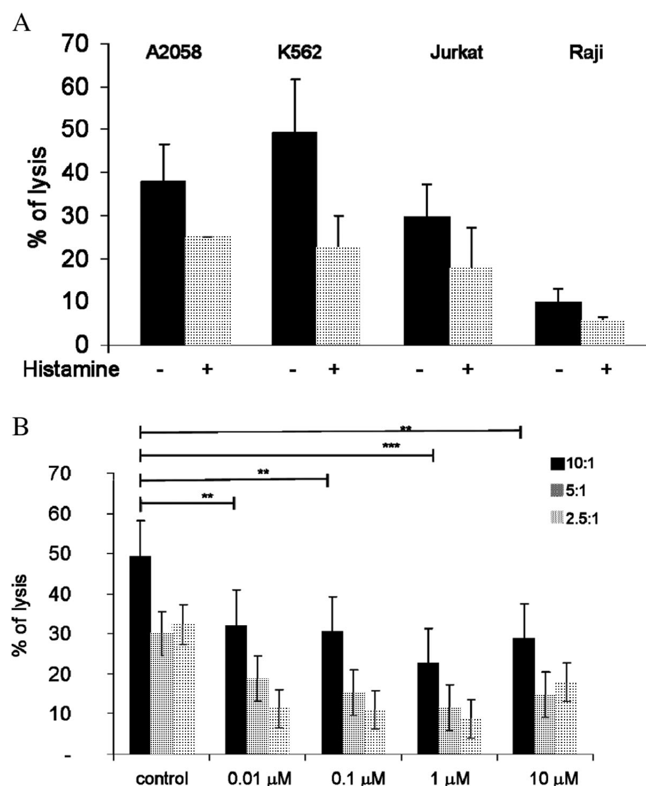

Figure 7 Cytotoxic activity γδ T cells from healthy donors towards tumour cell lines. (A) γδ T cells isolated from healthy donors were co-cultured with different tumour cell lines and the spontaneous cytolytic capacity was determined. (B) γδ T cells were stimulated with the indicated concentrations of histamine (0.01 μM–10 μM) and cytotoxicity against the chronic myeloid tumour cell line K562 was analysed. Cells were co-cultured in different effector: target ratios as indicated, on the right (E : T ratios: 10:1, 5:1 or 2.5:1). Data are means ± SEM (*n* = 3).

alone inhibited cytotoxicity by more than 50% compared with the histamine-untreated control cells. This inhibitory effect of the cholera toxin was further enhanced by histamine.

Discussion

Since its discovery in 1910, histamine has been regarded as one of the most important mediators in allergy and inflammation and is known to be involved in smooth muscle-stimulating, vasodepressor action and its involvement during anaphylaxis (Dale and Laidlaw, 1910). Although histamine is located in most body tissues, it is highly concentrated in the lungs, skin and gastrointestinal tract (Dunford *et al.*, 2006), where it has been shown to regulate gastric acid secretion in the stomach and neurological transmitter functions (Haas *et al.*, 2008; Ohtsu, 2008). In the central nervous system, histamine is involved in regulating drinking, body temperature, blood pressure and perceiving pain (Arrange *et al.*, 1983; Hill *et al.*, 1997). Moreover, histamine has also been described as an autocrine/paracrine or exogenous growth factor for cancer cells, e.g. malignant melanomas and leukaemia cells. In the case of chronic myeloid leukaemia, the secretion of histamine is the consequence of a leukaemia-specific oncogene (Aichberger *et al.*, 2006). To better understand the role of histamine in the crosstalk between immune cells and tumour cells, we performed studies and co-culture experiments with human γδ T cells, isolated from peripheral blood.

By demonstrating that histamine stimulates actin polymerization and Ca^{2+} transients in a concentration-dependent manner and migration in a typical bell-shaped concentration curve, we showed that the increase in intracellular Ca^{2+} is due to mobilization from intracellular stores, inasmuch as it is insensitive to chelation of extracellular Ca^{2+}. A long-lasting migration response requires a continuous interaction between migration-inducing ligands and their cell surface receptors to induce continuous cell activation of the 'cell motor' via actin polymerization. Therefore, a gradient of ligands,

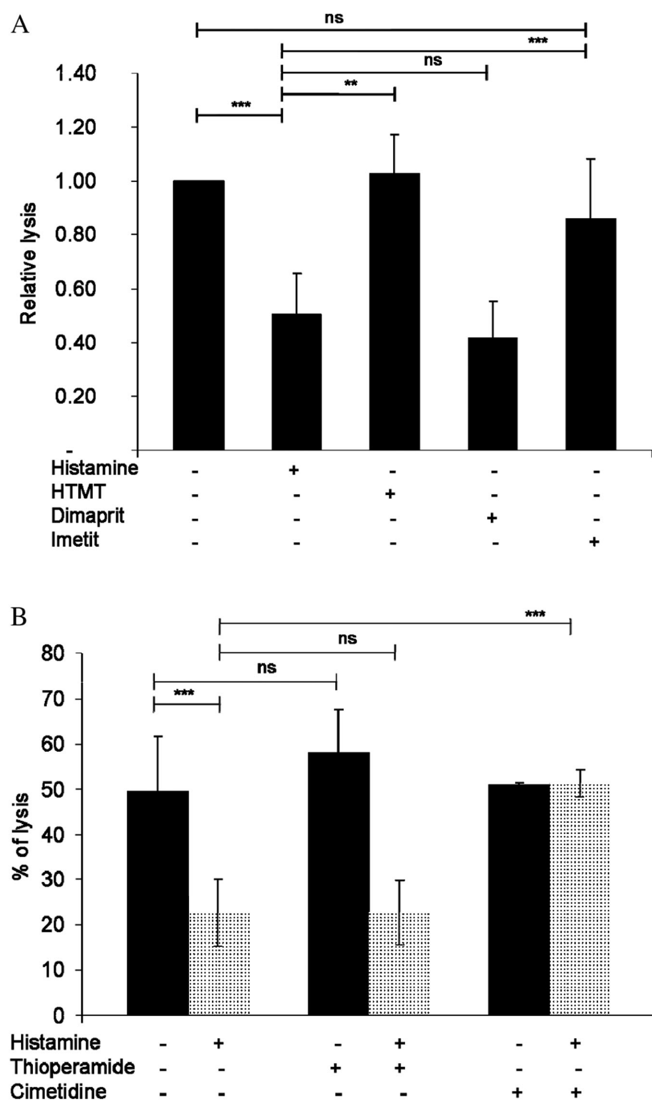

Figure 8 Dimaprit inhibits the cytotoxic activity of human γδ T cells. (A) γδ T cells were stimulated with 1 μM histamine, 10 μM H_1 receptor agonist HTMT, 10 μM H_2 receptor agonist dimaprit and 0.01 μM H_3 receptor agonist imetit and co-cultured with human myeloid cell line K562 in order to analyse the cytolytic activity. (B) γδ T cells were stimulated with 1 μM histamine in the presence or absence of 10 μM H_4 receptor antagonist thioperamide, or 10 μM H_2 receptor antagonist cimetidine and co-cultured with the human myeloid cell line K562 to analyse cytolytic activity. Data are means ± SEM (n = 3) (***$P < 0.0001$; **$P > 0.005$). ns, not significant.

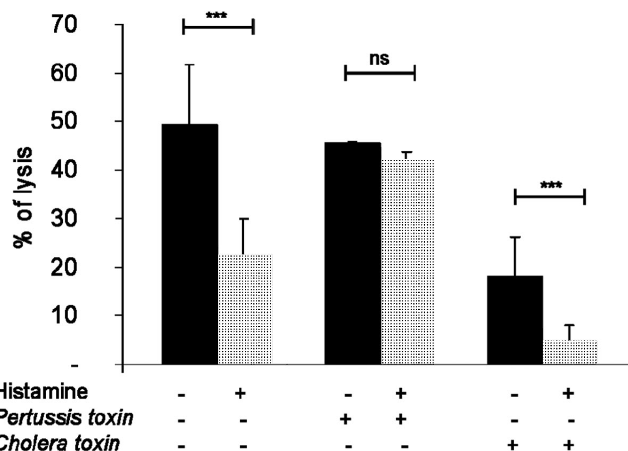

Figure 9 Cytotoxic activity of human γδ T cells against tumour cells K562 is dependent on G_s proteins. γδ T cells were pre-incubated with or without cholera toxin (0.5 μg·mL^{-1}) or *Pertussis* toxin (100 ng·mL^{-1}) for 1 h at 37°C. Thereafter, γδ T cells were stimulated or not with histamine and co-cultured with K562 cells. Data are means ± SEM (n = 3) (***$P < 0.0001$). ns, not significant.

continuously available cell surface receptors and a very sensitive signal transduction mechanism are necessary to transmit the external signal to the internal processes leading to cellular movement. Thus, a low concentration of chemotactic ligands can activate and direct the cell over a long period of time. At high ligand concentrations, the receptors at the cell surface are very quickly occupied and consequently desensitized as well as internalized via endocytosis. In this case, the CI is low because ligands find no functional receptors at the cell surface and are not able to induce movement until either novel transcriptionally regulated receptors are synthesized or the internalized receptors are recycled (Tranquillo *et al.*, 1988).

Histamine is a ligand for different G protein-coupled receptors. In order to demonstrate the participation of G_i proteins in these stimulated cell responses, experiments with *Pertussis* toxin were performed. This toxin selectively uncouples G_i proteins from the intracellular sites of receptors by ADP-ribosylation. Pretreating γδ T cells with *Pertussis* toxin blocked histamine-induced actin polymerization, Ca^{2+} transients and migration in γδ T cells, implying the involvement of G_i proteins in these cell responses. Principally, histamine binds to different receptor subtypes, H_1, H_2, H_3 and H_4 receptors. In the present work, RT-PCR revealed mRNA expression of histamine H_1, H_2 and H_4 receptors, but not for H_3 receptors, in γδ T cells. Moreover, expression of H_1, H_2 and H_4 receptor proteins was shown by Western blot analysis. The involvement of the different receptors in these cell responses was dissected using specific receptor agonists and antagonists. Our experiments revealed that histamine regulates actin polymerization, Ca^{2+} transients and chemotaxis via H_4 receptors, but provided no evidence for the involvement of H_1, H_2 or H_3 receptors in these cell responses. This receptor-isoform-specific cell regulation is consistent with reports in eosinophils, mast cells and NK cells (Hofstra *et al.*, 2003; Damaj *et al.*, 2007). Therefore one can assume that histamine H_4 receptors in γδ T cells activate *Pertussis* toxin-sensitive, heterotrimeric G_i proteins, which in turn dissociate into the GTP-a subunit and free βγ dimers. The latter activates phospholipase $C\beta_2$ (Camps *et al.*, 1992). This enzyme cleaves phosphatidylinositol (4,5)-bisphosphate into diacylglycerol and the inositol trisphosphate, which mobilizes Ca^{2+} from intracellular stores (Berridge and Imine, 1989). In leukocytes, G_i proteins regulate the reorganization of the actin cytoskeleton independently of activated phospholipase C (Stossel, 1989). These G_i protein-coupled signalling pathways are essential components of migration response in different subtypes of leukocytes (Hauert *et al.*, 2002).

Unlike classical chemotaxis-mediating receptors, such as chemokine receptors or complement C5a receptors, the coupling of different types of histamine receptors is pleiotropic,

including interaction of H_2 receptors with G_s proteins with consequent activation of adenylyl cyclase. Our cell studies combining histamine and selective receptor agonists or antagonists showed enhanced cAMP levels and, H_2 receptor activation in γδ T cells. In different subtypes of leukocytes, e.g. NK cells and $CD8^+$ T cells, the cytotoxicity response by cAMP has been reported to be inhibited (Wang *et al.*, 1995). Our data show that the spontaneous cytolytic activity of human γδ T cells was prevented by histamine. Neither HTMT, nor imetit nor thioperamide, altered the spontaneous cytolytic capacity of γδ T cells, but it was inhibited by dimaprit, suggesting that H_2 receptors may be involved in the inhibitory effect of histamine on the cytotoxicity of γδ T cells in human peripheral blood. Moreover, it has been shown that cholera toxin impairs cytotoxicity in αβ T lymphocytes and NK cells (Sugawara *et al.*, 1993). Consistent with an earlier report (Sugawara *et al.*, 1993), we found that the G_s protein activator cholera toxin inhibited the spontaneous cytotoxicity of γδ T cells, enhancing cAMP levels.

Infiltration by lymphocytes, macrophages, mast cells and neutrophils is a hallmark of inflammatory, defence and tissue repair reactions, which are often present in tumours. Various types of tumour-infiltrating macrophages and lymphocytes are considered as potential effectors of anti-tumour immunity and may interfere with tumour expression (Rosenberg, 2001). In this study, we have shown that histamine, which is present in the inflammatory and neoplastic microenvironment, induced the migration of human peripheral blood γδ T cells. In contrast, the spontaneous cytolytic effect of γδ T cells was prevented by histamine (Lazar-molnar *et al.*, 2000; Sonobe *et al.*, 2004). Neither HTMT, nor imetit nor thioperamide, altered the spontaneous cytolytic effect of γδ T cells, but it was inhibited by dimaprit, suggesting that H_2 receptors may be involved in the inhibitory effect of histamine on cytotoxicity of human peripheral blood γδ T cells. Our data suggest that histamine contributes to the escape of tumour cells from immunological surveillance.

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Conflict of interests

None.

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